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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:		)	
		)	
Applicant	: Ruoping Chen, et al.	)	
		)	TC /A.U. : 1646
Appl. No.	: 10/723,955	)	
		)	
Filed	: November 26, 2003	)	
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Pub. No.	**: US 2004/0110238 A1**	)	
		)	
Published	**: June 10, 2004**	)	
		)	
Title	: CONSTITUTIVELY ACTIVATED HUMAN G PROTEIN COUPLED RECEPTORS	)	

Mail Stop PGPUB
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CORRECTED APPLICATION PUBLICATION

Applicants respectfully request that the following corrections be made to Publication No.: US 2004/0110238-A1, published on June 10, 2004. Each of the proposed corrections is necessitated by printing errors made by the PTO. Accordingly, no fee is required.

1. Printing Error in Example 4

On page 17, in Example 4, sixth line of paragraph [0233], of the published application, please make the correction indicated on the attached marked-up version of page 17. This error is believed to be material to understanding the CRE-Luc Reporter Assay. A copy of the corresponding page of the specification as filed (Ser. No. 10/723,955), showing Example 4 as filed, has also been provided.

2. Other Printing Errors

Applicants respectfully request correction of the following additional PTO printing errors in the published application. The specific corrections to the published application are identified in the "Correction Chart for US 2004/0110238 A1" below. Copies of pages 2, 13, 15 and 17 of the published application showing the location of the errors, and the corresponding pages of the 10/723,955 application are also provided. The errors in the published application pages are identified by specific numbers, and are listed accordingly in the below Correction Chart.

Correction Chart for US 2004/0110238 A1

Printing error in the published application, number:	Corrections requested	Page, line and paragraph number of the error in the published application.	Page and line number of the originally filed application where the information was corrected submitted.
1	replace "**FIG. 1**" with --Figure 1--	page 2, fifth line of paragraph [0010]	page 4, line 3
2	for SEQ ID NO:85: replace "CAA" with --CAGA--	page 13, Table F	page 30, Table F

Publ. No.: US 2004/0110238 A1
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Please feel free to call Applicants if there are any questions.

Respectfully submitted,

ARENA PHARMACEUTICALS, INC.

Date: August 4, 2004 By: _____

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail, on the Date of Deposit shown above, postage prepaid and in an envelope addressed to the Mail Stop PGPUB; Commissioner for Patents; P. O. Box 1450; Alexandria, VA 22313-1450.

_____
Signature

C H McClure
Typed or Printed Name of Person Signing Certificate

Publ. No.: US 2004/0110238 A1
Published: June 10, 2004

	replace "1225K" with --I225K--	page 13, Table F	page 30, Table F
4	insert a comma between "orientation" and "mutation"	page 15, Table H	page 34, Table H
5	for SEQ ID NO:119: replace "CGGCTGG" with --GTCA--	page 15, Table H	page 34, Table H
6	for SEQ ID NO:122: replace "CCGCC" with --CCAGCC--	page 15, Table H	page 34, Table H
7	replace "1001 μl" with --100 μl— (Correction to Example 4 as discussed above)	Page 17, sixth line of paragraph [0233]	page 39, line 10

SUMMARY OF THE INVENTION

[0008] Disclosed herein are non-endogenous versions of endogenous, human GPCRs and uses thereof.

[0009] The present invention relates to a human T-cell death-associated gene receptor (TDAG8). The deletion of self-reactive immature T-cells in the thymus is mediated by apoptosis upon T-cell receptor interaction. Apoptosis is characterized by a rapid collapse of the nucleus, extreme chromatin condensation, DNA fragmentation, and shrinkage of cells, and it is often dependent on the synthesis of new sets of RNA and protein. (see, Choi et al., 168 Cellular Immun. 78 (1996)). There is a strong correlation between apoptosis and TDAG8; i.e., an increase in apoptosis results in an increase in the expression of TDAG8. Id. However, it is unknown whether an increase in TDAG8 expression causes T-cell mediated apoptosis, or if such expression is a result of such apoptosis.

[0010] The endogenous ligand for TDAG8 is unknown and is thus considered an orphan GPCR having an open reading frame of 1,011 bp encoding a 337 amino acid protein. (TDAG8 was cloned and sequenced in 1998. Kyaw, H. et al, 17 DNA Cell Biol. 493 (1998); see FIG. 1 of Kyaw for nucleic and deduced amino acid sequences.). Human TDAG8 is reported to be homologous to murine TDAG8. Human TDAG8 is expressed in the liver and in lymphoid tissues, including peripheral blood leukocytes, spleen, lymph nodes and thymus. TDAG8 is also reported to be localized to chromosome 14q31-32.1. Id.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] **FIG. 1** is a representation of 8×CRE-Luc reporter plasmid (see, Example 4(c)3.)

[0012] **FIGS. 2A and 2B** are graphic representations of the results of ATP and ADP binding to endogenous TDAG8 (2A) and comparisons in serum and serum free media (2B).

[0013] **FIG. 3** is a graphic representation of the comparative signaling results of CMV versus the GPCR Fusion Protein H9(F236K):Gsα.

[0014] FIGS. 4A-4B is a representation of a dose response curve for endogenous, constitutively active TDAG8 ("TDAG8 WT") in 293 cell-based cAMP assay. **FIG. 4A** shows ATP binding to "TDAG8 WT" at an EC50 value of 500 μM, while **FIG. 4B** shows ADP binding to "TDAG8 WT" at an EC50 value of 700 μM.

[0015] FIGS. 5A-5B provides graphic results of comparative analysis of endogenous TDAG8 ("WT") versus non-endogenous, constitutively active TDAG8 ("I225K") (control is designated "CMV") in 293 (5A) and 293T (5B) cells.

[0016] **FIG. 6** is a reproduction of results of a tissue distribution of TDAG8 against various tissue-source mRNA's.

DETAILED DESCRIPTION

[0017] The scientific literature that has evolved around receptors has adopted a number of terms to refer to ligands having various effects on receptors. For clarity and consistency, the following definitions will be used throughout this patent document. To the extent that these definitions conflict with other definitions for these terms, the following definitions shall control:

[0018] AGONISTS shall mean materials (e.g., ligands, candidate compounds) that activate the intracellular response when they bind to the receptor, or enhance GTP binding to membranes.

[0019] AMINO ACID ABBREVIATIONS used herein are set out in Table A:

TABLE A

ALANINE	ALA	A
ARGININE	ARG	R
ASPARAGINE	ASN	N
ASPARTIC ACID	ASP	D
CYSTEINE	CYS	C
GLUTAMIC ACID	GLU	E
GLUTAMINE	GLN	Q
GLYCINE	GLY	G
HISTIDINE	HIS	H
ISOLEUCINE	ILE	I
LEUCINE	LEU	L
LYSINE	LYS	K
METHIONINE	MET	M
PHENYLALANINE	PHE	F
PROLINE	PRO	P
SERINE	SER	S
THREONINE	THR	T
TRYPTOPHAN	TRP	W
TYROSINE	TYR	Y
VALINE	VAL	V

[0020] PARTIAL AGONISTS shall mean materials (e.g., ligands, candidate compounds) that activate the intracellular response when they bind to the receptor to a lesser degree/extent than do agonists, or enhance GTP binding to membranes to a lesser degree/extent than do agonists.

[0021] ANTAGONIST shall mean materials (e.g., ligands, candidate compounds) that competitively bind to the receptor at the same site as the agonists but which do not activate the intracellular response initiated by the active form of the receptor, and can thereby inhibit the intracellular responses by agonists or partial agonists. ANTAGONISTS do not diminish the baseline intracellular response in the absence of an agonist or partial agonist.

[0022] CANDIDATE COMPOUND shall mean a molecule (for example, and not limitation, a chemical compound) that is amenable to a screening technique. Preferably, the phrase "candidate compound" does not include compounds which were publicly known to be compounds selected from the group consisting of inverse agonist, agonist or antagonist to a receptor, as previously determined by an indirect identification process ("indirectly identified compound"); more preferably, not including an indirectly identified compound which has previously been determined to have therapeutic efficacy in at least one mammal; and, most preferably, not including an indirectly identified compound which has previously been determined to have therapeutic utility in humans.

[0023] COMPOSITION means a material comprising at least one component; a "pharmaceutical composition" is an example of a composition.

[0024] COMPOUND EFFICACY shall mean a measurement of the ability of a compound to inhibit or stimulate receptor functionality, as opposed to receptor binding affinity. Exemplary means of detecting compound efficacy are disclosed in the Example section of this patent document.

TABLE F-continued

Receptor Identifier	Codon Mutation	Lysine Mutagenesis (SEQ.ID.NO.) 5'-3' orientation, mutation sequence underlined	Selection Marker (SEQ.ID.NO.) 5'-3' orientation
hGPR38	V297K	GGCCACCGCCAAGCAAAC GCGTCCTGCTG (85)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAGT (86)
hCCKB	V332K	alternative approach; see below	alternative approach; see below
hTDAG8	I225K	GGAAAAGAAGAGAATCAA AAAACTACTTGTCAGCATC (87)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAGT (88)
hH9	F236K	GCTGAGGTTCGCAATAAAC TAACCATGTTTGTG (143)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAGT (144)
hMC4	A244K	GCCAATATGAAGGGAAAA ATTACCTTGACCATC (137)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAGT (138)

[0168] The non-endogenous human GPCRs were then sequenced and the derived and verified nucleic acid and amino acid sequences are listed in the accompanying "Sequence Listing" appendix to this patent document, as summarized in Table G below:

TABLE G

Non Endogenous Human GPCR	Nucleic Acid Sequence Listing	Amino Acid Sequence Listing
hRUP4 (V272K)	SEQ. ID. NO.: 127	SEQ. ID. NO.: 128
hAT1 (see alternative approaches below)	(see alternative approaches below)	(see alternative approaches, below)
hGPR38 (V297K)	SEQ. ID. NO.: 129	SEQ. ID. NO.: 130
hCCKB (V332K)	SEQ. ID. NO.: 131	SEQ. ID. NO.: 132
HTDAG8 (I225K)	SEQ. ID. NO.: 133	SEQ. ID. NO.: 134
hH9 (F236K)	SEQ. ID. NO.: 141	SEQ. ID. NO.: 142
hMC4 (A244K)	SEQ. ID. NO.: 135	SEQ. ID. NO.: 136

[0169] 2. Alternative Approaches For Creation of Non-Endogenous Human GPCRs

[0170] a. AT1

[0171] 1. F239K Mutation

[0172] Preparation of a non-endogenous, constitutively activated human AT1 receptor was accomplished by creating an F239K mutation (see, SEQ.ID.NO.: 89 for nucleic acid sequence, and SEQ.ID.NO.: 90 for amino acid sequence). Mutagenesis was performed using Transformer Site-Directed Mutagenesis™ Kit (Clontech) according to the to manufacturer's instructions. The two mutagenesis primers were used, a lysine mutagenesis oligonucleotide (SEQ.ID.NO.: 91) and a selection marker oligonucleotide (SEQ.ID.NO.: 92), which had the following sequences:

(SEQ.ID.NO.:91)
5'-CCAAGAAATGATGATATTAAAAAGATAATTATGGC-3'

(SEQ.ID.NO.:92)
5'-CTCCTTCGGTCCTCCTATCGTTGTCAGAAGT-3'

[0173] respectively.

[0174] //

[0175] 2. N111A Mutation

[0176] Preparation of a non-endogenous human AT1 receptor was also accomplished by creating an N111A mutation (see, SEQ.ID.NO.:93 for nucleic acid sequence, and SEQ.ID.NO.: 94 for amino acid sequence). Two PCR reactions were performed using pfu polymerase (Stratagene) with the buffer system provided by the manufacturer, supplemented with 10% DMSO, 0.25 μM of each primer, and 0.5 mM of each 4 nucleotides. The 5' PCR sense primer used had the following sequence:

[0177] 5'-CCCAAGCTTCCCCAGGTG-TATTTGAT-3' (SEQ.ID.NO.: 95)

[0178] and the antisense primer had the following sequence:

[0179] 5'-CCTGCAGGCGAAACTGACTCTGGCT-GAAG-3' (SEQ.ID.NO.: 96).

[0180] The resulting 400 bp PCR fragment was digested with HindIII site and subcloned into HindIII-SmaI site of pCMV vector (5' construct). The 3' PCR sense primer used had the following sequence:

[0181] 5'-CTGTACGCTAGTGTGTTTCTACT-CACGTGTCTCAGCATTGAT-3' (SEQ.ID.NO.: 97)

[0182] and the antisense primer had the following sequence:

[0183] 5'-GTTGGATCCACATAATGCATTTTCTC-3' (SEQ.ID.NO.: 98)

[0184] The resulting 880 bp PCR fragment was digested with BamHI and inserted into Pst (blunted by T4 poly-

and EcoRI and cloned into HindIII-EcoRI site of pCMV expression vector. (See, SEQ.ID.NO.: 111).

[0211] 3. QuikChange™ Site-Directed™ Mutagenesis

[0212] Preparation of non-endogenous human GPCRs can also be accomplished by using QuikChange™ Site-Directed™ Mutagenesis Kit (Stratagene, according to manufacturer's instructions). Endogenous GPCR is preferably used as a template and two mutagenesis primers utilized, as well as, most preferably, a lysine mutagenesis oligonucleotide and a selection marker oligonucleotide (included in kit). For convenience, the codon mutation incorporated into the human GPCR and the respective oligonucleotides are noted, in standard form (Table H):

TABLE H

Receptor Identifier	Codon Mutation	Lysine Mutagenesis (SEQ.ID.NO.) 5'-3' orientation mutation underlined	Selection Marker (SEQ.ID.NO.) 5'-3' orientation
hCHN3	S284K	ATGGAGAAAAGAATCAAAAGAA TGTTCTATATA (115)	TATATAGAACATTCTTTT GATTCTTTTCTCCAT (116)
hCHN6	L352K	CGCTCTCTGGCCTTGAAGCGCAC GCTCAGC (117)	GCTGAGCGTGCGCTTCA AGGCCAGAGAGCG (118)
hCHN8	N235K	CCCAGGAAAAAGGTGAACGGCTGG AAGTTTTC (119)	GAAAACTTTGACTTTCAC CTTTTTCCTGGG (120)
hCHN9	G223K	GGGGCGCGGGTGAAACGGCTGG TGAGC (121)	GCTCACCGCCGTTTCAC CCGCGCCCC (122)
hCHN10	L231K	CCCCTTGAAAAGCCTAAGAACTT GGTCATC (123)	GATGACCAAGTTCTTAG GCTTTTCAAGGGG (124)

Example 3

[0213] Receptor Expression

[0214] Although a variety of cells are available to the art for the expression of proteins, it is most preferred that mammalian cells be utilized. The primary reason for this is predicated upon practicalities, i.e., utilization of, e.g., yeast cells for the expression of a GPCR, while possible, introduces into the protocol a non-mammalian cell which may not (indeed, in the case of yeast, does not) include the receptor-coupling, genetic-mechanism and secretary pathways that have evolved for mammalian systems—thus, results obtained in non-mammalian cells, while of potential use, are not as preferred as that obtained from mammalian cells. Of the mammalian cells, COS-7, 293 and 293T cells are particularly preferred, although the specific mammalian cell utilized can be predicated upon the particular needs of the artisan.

[0215] On day one, 1×10^7 293T cells per 150 mm plate were plated out. On day two, two reaction tubes were prepared (the proportions to follow for each tube are per plate): tube A was prepared by mixing 20 μg DNA (e.g., pCMV vector; pCMV vector with receptor cDNA, etc.) in 1.2 ml serum free DMEM (Irvine Scientific, Irvine, Calif.); tube B was prepared by mixing 120 μl lipofectamine (Gibco BRL) in 1.2 ml serum free DMEM. Tubes A and B were admixed by inversions (several times), followed by incubation at room temperature for 30-45 min. The admixture is referred to as the "transfection mixture". Plated 293T cells were washed with 1×PBS, followed by addition of 10 ml serum free DMEM. 2.4 ml of the transfection mixture were added to the cells, followed by incubation for 4 hrs at 37° C./5% CO_2. The transfection mixture was removed by aspiration, followed by the addition of 25 ml of DMEM/10% Fetal Bovine Serum. Cells were incubated at 37° C./5% CO_2. After 72 hr incubation, cells were harvested and utilized for analysis.

Example 4

[0216] Assays for Determination of Constitutive Activity of Non-Endogenous GPCRs

[0217] A variety of approaches are available for assessment of constitutive activity of the non-endogenous human GPCRs. The following are illustrative; those of ordinary skill in the art are credited with the ability to determine those techniques that are preferentially beneficial for the needs of the artisan.

[0218] 1. Membrane Binding Assays: [^{35}S]GTPγS Assay

[0219] When a G protein-coupled receptor is in its active state, either as a result of ligand binding or constitutive activation, the receptor couples to a G protein and stimulates the release of GDP and subsequent binding of GTP to the G protein. The alpha subunit of the G protein-receptor complex acts as a GTPase and slowly hydrolyzes the GTP to GDP, at which point the receptor normally is deactivated. Constitutively activated receptors continue to exchange GDP for GTP. The non-hydrolyzable GTP analog, [^{35}S]GTPγS, can be utilized to demonstrate enhanced binding of [^{35}S]GTPγS to membranes expressing constitutively activated receptors. The advantage of using [^{35}S]GTPγS binding to measure constitutive activation is that: (a) it is generically applicable to all G protein-coupled receptors; (b) it is proximal at the membrane surface making it less likely to pick-up molecules which affect the intracellular cascade.

the activation of genes containing AP1 elements in their promoter. A Pathdetect™ AP-1 cis-Reporting System (Stratagene, Catalogue #219073) can be utilized following the protocol set forth above with respect to the CREB reporter assay, except that the components of the calcium phosphate precipitate were 410 ng pAP1-Luc, 80 ng pCMV-receptor expression plasmid, and 20 ng CMV-SEAP.

[0232] 3. CRE-Luc Reporter Assay

[0233] 293 and 293T cells are plated-out on 96 well plates at a density of 2×10^4 cells per well and were transfected using Lipofectamine Reagent (BRL) the following day according to manufacturer instructions. A DNA/lipid mixture is prepared for each 6-well transfection as follows: 260 ng of plasmid DNA in 100 μl of DMEM were gently mixed with 2 μl of lipid in 100 μl of DMEM (the 260 ng of plasmid DNA consisted of 200 ng of a 8×CRE-Luc reporter plasmid (see below and **FIG. 1** for a representation of a portion of the plasmid), 50 ng of pCMV comprising endogenous receptor or non-endogenous receptor or pCMV alone, and 10 ng of a GPRS expression plasmid (GPRS in pcDNA3 (Invitrogen)). The 8×CRE-Luc reporter plasmid was prepared as follows: vector SRIF-β-gal was obtained by cloning the rat somatostatin promoter (−71/+51) at BglV-HindIII site in the pβgal-Basic Vector (Clontech). Eight (8) copies of cAMP response element were obtained by PCR from an adenovirus template AdpCF126CCRE8 (see, 7 *Human Gene Therapy* 1883 (1996)) and cloned into the SRIF-β-gal vector at the Kpn-BglV site, resulting in the 8×CRE-β-gal reporter vector. The 8×CRE-Luc reporter plasmid was generated by replacing the beta-galactosidase gene in the 8×CRE-β-gal reporter vector with the luciferase gene obtained from the pGL3-basic vector (Promega) at the HindIII-BamHI site. Following 30 min. incubation at room temperature, the DNA/lipid mixture was diluted with 400 μl of DMEM and 100 μl of the diluted mixture was added to each well. 100 μl of DMEM with 10% FCS were added to each well after a 4 hr incubation in a cell culture incubator. The following day the transfected cells were changed with 200 μl/well of DMEM with 10% FCS. Eight (8) hours later, the wells were changed to 100 μl /well of DMEM without phenol red, after one wash with PBS. Luciferase activity were measured the next day using the LucLite™ reporter gene assay kit (Packard) following manufacturer instructions and read on a 1450 MicroBeta™ scintillation and luminescence counter (Wallac).

[0234] 4. SRF-Luc Reporter Assay

[0235] One method to detect Gq stimulation depends on the known property of Gq-dependent phospholipase C to cause the activation of genes containing serum response factors in their promoter. A Pathdetect™ SRF-Luc-Reporting System (Stratagene) can be utilized to assay for Gq coupled activity in, e.g., COS7 cells. Cells are transfected with the plasmid components of the system and the indicated expression plasmid encoding endogenous or non-endogenous GPCR using a Mammalian Transfection™ Kit (Stratagene, Catalogue #200285) according to the manufacturer's instructions. Briefly, 410 ng SRF-Luc, 80 ng pCMV-receptor expression plasmid and 20 ng CMV-SEAP (secreted alkaline phosphatase expression plasmid; alkaline phosphatase activity is measured in the media of transfected cells to control for variations in transfection efficiency between samples) are combined in a calcium phosphate precipitate as per the manufacturer's instructions. Half of the precipitate is equally distributed over 3 wells in a 96-well plate, kept on the cells in a serum free media for 24 hours. The last 5 hours the cells are incubated with 1 μM Angiotensin, where indicated. Cells are then lysed and assayed for luciferase activity using a Luclite™ Kit (Packard, Cat. #6016911) and "Trilux 1450 Microbeta" liquid scintillation and luminescence counter (Wallac) as per the manufacturer's instructions. The data can be analyzed using GraphPad Prism™ 2.0a (GraphPad Software Inc.).

[0236] 5. Intracellular IP_3 Accumulation Assay

[0237] On day 1, cells comprising the receptors (endogenous and/or non-endogenous) can be plated onto 24 well plates, usually 1×10^5 cells/well (although his umber can be optimized. On day 2 cells can be transfected by firstly mixing 0.25 ug DNA in 50 ul serum free DMEM/well and 2 ul lipofectamine in 50 μl serumfree DMEM/well. The solutions are gently mixed and incubated for 15-30 min at room temperature. Cells are washed with 0.5 ml PBS and 400 μl of serum free media is mixed with the transfection media and added to the cells. The cells are then incubated for 3-4 hrs at 37° C./5% CO_2 and then the transfection media is removed and replaced with 1 ml/well of regular growth media. On day 3 the cells are labeled with ^{3}H-myo-inositol. Briefly, the media is removed and the cells are washed with 0.5 ml PBS. Then 0.5 ml inositol-free/serum free media (GIBCO BRL) is added/well with 0.25 μCi of ^{3}H-myo-inositol/well and the cells are incubated for 16-18 hrs o/n at 37° C./5% CO_2 . On Day 4 the cells are washed with 0.5 ml PBS and 0.45 ml of assay medium is added containing inositol-free/serum free media 10 μM pargyline 10 mM lithium chloride or 0.4 ml of assay medium and 50 ul of 10× ketanserin (ket) to final concentration of 10 μM. The cells are then incubated for 30 min at 37° C. The cells are then washed with 0.5 ml PBSand 200 ul of fresh/icecold stop solution (1M KOH; 18 mM Na-borate; 3.8 mM EDTA) is added/well. The solution is kept on ice for 5-10 min or until cells were lysed and then neutralized by 200 μl of fresh/ice cold neutralization sol. (7.5% HCL). The lysate is then transferred into 1.5 ml eppendorf tubes and 1 ml of chloroform/methanol (1:2) is added/tube. The solution is vortexed for 15 sec and the upper phase is applied to a Biorad AG1-X8™ anion exchange resin (100-200 mesh). Firstly, the resin is washed with water at 1:1.25 W/V and 0.9 ml of upper phase is loaded onto the column. The column is washed with 10 mls of 5 mM myo-inositol and 10 ml of 5 mM Na-borate/60 mM Na-formate. The inositol tris phosphates are eluted into scintillation vials containing 10 ml of scintillation cocktail with 2 ml of 0.1 M formic acid/1 M ammonium formate. The columns are regenerated by washing with 10 ml of 0.1 M formic acid/3M ammonium formate and rinsed twice with dd H_2O and stored at 4° C. in water.

[0238] Exemplary results are presented below in Table I and, in the case of hTDAG8, also in histogram form in **FIGS. 5A** (293 cells) and **5B** (293T cells).

The endogenous ligand for TDAG8 is unknown and is thus considered an orphan GPCR having an open reading frame of 1,011 bp encoding a 337 amino acid protein. (TDAG8 was cloned and sequenced in 1998. Kyaw, H. et al, 17 DNA Cell Biol. 493 (1998); *see* Figure 1 of Kyaw for nucleic and deduced amino acid sequences.). Human TDAG8 is reported to be homologous to murine TDAG8. Human TDAG8 is expressed in the liver and in lymphoid tissues, including peripheral blood leukocytes, spleen, lymph nodes and thymus. TDAG8 is also reported to be localized to chromosome 14q31-32.1. *Id.*

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a representation of 8XCRE-Luc reporter plasmid (*see*, Example 4(c)3.)

Figures 2A and **2B** are graphic representations of the results of ATP and ADP binding to endogenous TDAG8 (2A) and comparisons in serum and serum free media (2B).

Figure 3 is a graphic representation of the comparative signaling results of CMV versus the GPCR Fusion Protein H9(F236K):Gsα.

Figures 4A-4B is a representation of a dose response curve for endogenous, constitutively active TDAG8 ("TDAG8 WT") in 293 cell-based cAMP assay. Figure 4A shows ATP binding to "TDAG8 WT" at an EC50 value of 500μM, while Figure 4B shows ADP binding to "TDAG8 WT" at an EC50 value of 700μM.

Figures 5A-5B provides graphic results of comparative analysis of endogenous TDAG8 ("WT") versus non-endogenous, constitutively active TDAG8 ("I225K") (control is designated "CMV") in 293 (5A) and 293T (5B) cells.

Figure 6 is a reproduction of results of a tissue distribution of TDAG8 against various tissue-source mRNA's.

DETAILED DESCRIPTION

The scientific literature that has evolved around receptors has adopted a number of terms to refer to ligands having various effects on receptors. For clarity and consistency, the following definitions will be used throughout this patent document. To the extent that these definitions conflict with other definitions for these terms, the following definitions shall control:

AGONISTS shall mean materials (*e.g.*, ligands, candidate compounds) that activate the intracellular response when they bind to the receptor, or enhance GTP binding to membranes.

TABLE F

Receptor Identifier	Codon Mutation	Lysine Mutagenesis (SEQ.ID.NO.) 5'-3' orientation, mutation sequence underlined	Selection Marker (SEQ.ID.NO.) 5'-3' orientation
hRUP4	V272K	CAGGAAGAAGAAACGAGC TGTCATTATGATGGTGACA GTG (83)	CACTGTCACCATCATAATG ACAGCTCGTTTCTTCTTCC TG (84)
hAT1	*see* below	alternative approach; *see* below	alternative approach; *see* below
hGPR38	V297K	GGCCACCGGCAGACCAAAC GCGTCCTGCTG (85)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAGT (86)
hCCKB	V332K	alternative approach; *see* below	alternative approach; *see* below
hTDAG8	I225K	GGAAAAGAAGAGAATCAA AAAACTACTTGTCAGCATC (87)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAGT (88)
hH9	F236K	GCTGAGGTTCGCAATAAAC TAACCATGTTTGTG (143)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAGT (144)
hMC4	A244K	GCCAATATGAAGGGAAAA ATTACCTTGACCATC (137)	CTCCTTCGGTCCTCCTATC GTTGTCAGAAGT (138)

The non-endogenous human GPCRs were then sequenced and the derived and verified nucleic acid and amino acid sequences are listed in the accompanying "Sequence Listing" appendix to this patent document, as summarized in Table G below:

TABLE G

Non Endogenous Human GPCR	Nucleic Acid Sequence Listing	Amino Acid Sequence Listing
hRUP4 (V272K)	SEQ.ID.NO.: 127	SEQ.ID.NO.: 128
hAT1 (*see* alternative approaches below)	(*see* alternative approaches below)	(*see* alternative approaches, below)
hGPR38 (V297K)	SEQ.ID.NO.: 129	SEQ.ID.NO.: 130
hCCKB (V332K)	SEQ.ID.NO.: 131	SEQ.ID.NO.: 132
HTDAG8 (I225K)	SEQ.ID.NO.: 133	SEQ.ID.NO.: 134
hH9 (F236K)	SEQ.ID.NO.: 141	SEQ.ID.NO.: 142
hMC4 (A244K)	SEQ.ID.NO.: 135	SEQ.ID.NO.: 136

2. Alternative Approaches For Creation of Non-Endogenous Human GPCRs

5'-CAGCAGCATGCGCTTCACGCGCTTCTTAGCCCAG-3' (SEQ.ID.NO.: 113).

The second PCR fragment (0.44kb) was amplified by using a sense primer comprising the V322K mutation:

5'-AGAAGCGCGTGAAGCGCATGCTGCTGGTGATCGTT-3' (SEQ.ID.NO.: 114) and SEQ.ID.NO.: 76.

The two resulting PCR fragments were then used as template for amplifying CCKB comprising V332K, using SEQ.ID.NO.: 75 and SEQ.ID.NO.: 76 and the above-noted system and conditions. The resulting 1.44kb PCR fragment containing the V332K mutation was digested with HindIII and EcoRI and cloned into HindIII-EcoRI site of pCMV expression vector. (*See,* SEQ.ID.NO.: 111).

3. QuikChange™ Site-Directed™ Mutagenesis

Preparation of non-endogenous human GPCRs can also be accomplished by using QuikChange™ Site-Directed™ Mutagenesis Kit (Stratagene, according to manufacturer's instructions). Endogenous GPCR is preferably used as a template and two mutagenesis primers utilized, as well as, most preferably, a lysine mutagenesis oligonucleotide and a selection marker oligonucleotide (included in kit). For convenience, the codon mutation incorporated into the human GPCR and the respective oligonucleotides are noted, in standard form (Table H):

TABLE H

Receptor Identifier	Codon Mutation	Lysine Mutagenesis (SEQ.ID.NO.) 5'-3' orientation, mutation underlined	Selection Marker (SEQ.ID.NO.) 5'-3' orientation
hCHN3	S284K	ATGGAGAAAAGAATCAAAAGAA TGTTCTATATA (115)	TATATAGAACATTCTTTT GATTCTTTTCTCCAT (116)
hCHN6	L352K	CGCTCTCTGGCCTTGAAGCGCAC GCTCAGC (117)	GCTGAGCGTGCGCTTCA AGGCCAGAGAGCG (118)
hCHN8	N235K	CCCAGGAAAAAGGTGAAAGTCA AAGTTTTC (119)	GAAAACTTTGACTTTCAC CTTTTTCCTGGG (120)
hCHN9	G223K	GGGGCGCGGGTGAAACGGCTGG TGAGC (121)	GCTCACCAGCCGTTTCAC CCGCGCCCC (122)
hCHN10	L231K	CCCCTTGAAAAGCCTAAGAACTT GGTCATC (123)	GATGACCAAGTTCTTAG GCTTTTCAAGGGG (124)

Example 3

RECEPTOR EXPRESSION

A Pathdetect™ AP-1 cis-Reporting System (Stratagene, Catalogue # 219073) can be utilized following the protocol set forth above with respect to the CREB reporter assay, except that the components of the calcium phosphate precipitate were 410 ng pAP1-Luc, 80 ng pCMV-receptor expression plasmid, and 20 ng CMV-SEAP.

3. CRE-LUC Reporter Assay

293 and 293T cells are plated-out on 96 well plates at a density of 2 x 10^4 cells per well and were transfected using Lipofectamine Reagent (BRL) the following day according to manufacturer instructions. A DNA/lipid mixture is prepared for each 6-well transfection as follows: 260ng of plasmid DNA in 100μl of DMEM were gently mixed with 2μl of lipid in 100μl of DMEM (the 260ng of plasmid DNA consisted of 200ng of a 8xCRE-Luc reporter plasmid (*see* below and Figure 1 for a representation of a portion of the plasmid), 50ng of pCMV comprising endogenous receptor or non-endogenous receptor or pCMV alone, and 10ng of a GPRS expression plasmid (GPRS in pcDNA3 (Invitrogen)). The 8XCRE-Luc reporter plasmid was prepared as follows: vector SRIF-β-gal was obtained by cloning the rat somatostatin promoter (-71/+51) at BglV-HindIII site in the pβgal-Basic Vector (Clontech). Eight (8) copies of cAMP response element were obtained by PCR from an adenovirus template AdpCF126CCRE8 (*see*, 7 *Human Gene Therapy* 1883 (1996)) and cloned into the SRIF-β-gal vector at the Kpn-BglV site, resulting in the 8xCRE-β-gal reporter vector. The 8xCRE-Luc reporter plasmid was generated by replacing the beta-galactosidase gene in the 8xCRE-β-gal reporter vector with the luciferase gene obtained from the pGL3-basic vector (Promega) at the HindIII-BamHI site. Following 30 min. incubation at room temperature, the DNA/lipid mixture was diluted with 400 μl of DMEM and 100μl of the diluted mixture was added to each well. 100 μl of DMEM with 10% FCS were added to each well after a 4hr incubation in a cell culture incubator. The following day the transfected cells were changed with 200 μl/well of DMEM with 10% FCS. Eight (8) hours later, the wells were changed to 100 μl /well of DMEM without phenol red, after one wash with PBS. Luciferase activity were measured the next day using the LucLite™ reporter gene assay kit (Packard) following manufacturer instructions and read on a 1450 MicroBeta™ scintillation and luminescence counter (Wallac).

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- ☐ Fee Transmittal Form
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Date	August 4, 2004

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